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Mixtures of aluminum and indium induce more than additive phenotypic and toxicogenomic responses in *Daphnia magna*

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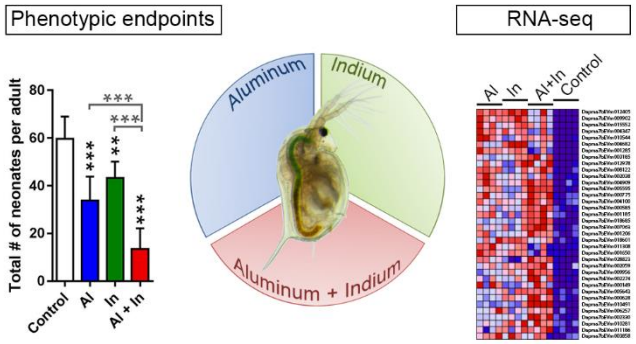


Abstract

Aquatic systems are contaminated by many metals but their effects as mixtures on organisms are not well understood. Here, we assessed effects of aluminum with fairly well-known modes of actions and indium, an understudied emerging contaminant from electronics, followed by studying equi-effective mixtures thereof. We report acute and adverse phenotypic effects in *Daphnia magna* adults and global transcriptomic effects employing RNA sequencing in neonates. The mixture induced more than additive activity in mortality, and in physiological effects, including growth and reproduction. Similarly, transcriptomic effects were more than additive, as indicated by a markedly higher number of 463 differentially expressed transcripts in the mixture and by distinct classes of genes assigned to several biological functions, including metabolic processes, suggesting depleted energy reserves, which may be responsible for the observed impaired reproduction and growth. A gene set enrichment analysis (GSEA) of *a priori* known response pathways for aluminum confirmed activation of distinct molecular pathways by indium. Our study is highlighting more than additive effects at the transcriptional and physiological level and is providing a state-of-the art approach to mixture analysis, which is important for risk assessment of these metals and metal mixtures.

Introduction

Aquatic organisms are continuously exposed to multiple chemicals from different sources in polluted environments. Among them are metals, including aluminum (Al) mobilized from bedrock in acidified waters and indium (In) originating from the production, use, and disposal of electronic devices.[1–3] In risk assessment, the joint activity associated with mixtures of chemicals are still not fully considered.[4] However, increasing evidence indicates that joint activity of mixtures matters and mixtures can even have more than additive effects.[5]

Mixture activity can be described by two concepts. Effects of chemical mixtures with a similar mode of action (MoA) such as binding to the same receptors are assessed using the concept of concentration addition (CA),[6] in which it is assumed that one compound can be replaced by an equal fraction of an equally effective concentration of another. Thus, each component of the mixture contributes to the combined effects in proportion to its concentration and individual potency. In case of compounds with dissimilar MoAs in a mixture, and thus, interacting with different target sites, the concept of independent action is applied.[7] Thus, joint toxic effects can be estimated multiplying the probabilities of responses.[8] Both concepts are applicable to metals, with the CA concept being the most conservative.[9–11] Both concepts rely on compound additivity in a mixture. However, mixtures can also lead to an overall more than additive (synergistic) or lower than additive (antagonistic) effect.[9] Synergistic interactions were previously found for endocrine active organic compounds *in vitro*[12,13] and *in vivo*[14,15] in fish and for metal mixtures in *Daphnia*[16–18] and may be explained by combined activated molecular pathways that converge, and thus, potentiate the response.

Here, we study the effects of binary mixtures of Al and In to better understand mixture interactions of trivalent metals and shed light on the barely known aquatic toxicity of the emerging contaminant In. In principal, these two trivalent metals may coexist and interactions may occur, as Al is commonly found in the aquatic environment. Contamination by In is more restricted to contamination sites, such as industrial production of electronics, but also to disposal and recycling sites of electronic waste due to its application in modern electronic equipment including smart phones, flat panel displays, and light emitting diodes (LEDs).[19] Particularly high concentrations are measured in lakes influenced by acidic deposition with up to 396.3 µg L^{-1} Al[20] and in river sediments near smelters with up to 75 mg kg^{-1} In[3]. Due to the prevalence of Al in acidified

69 waters but also in manufacturing and as in industrial catalysts,[21] the toxic potential of Al has been studied. The
70 MoAs include immune system responses,[22,23] oxidative stress,[24] and hypoxia and apoptosis[22] among others. In
71 contrast, ecotoxicological effects of In are poorly known.[25,26]

72 In our study, we applied the CA concept to assess the joint activity of Al and In. Our rationale for
73 applying the CA concept is the fact, that first, there are clear dose-response relationships in the endpoint
74 (mortality) on which our equi-effective concentrations is based upon. This is necessary in contrast to the IA
75 concept. Second, In and Al are trivalent metals and thus might have a similar uptake into cells of *Daphnia*.
76 Third, we hypothesized *a priori* similar MoAs of Al and In. Moreover, when assessing CA responses of binary
77 mixtures at phenotypic and transcriptional levels, no previous knowledge other than an effective
78 concentration for both compounds is needed setting the stage for including emerging compounds in mixture
79 toxicity assessment.

80 The aim of our present study was to assess the mixture activity of binary mixtures of Al and In for
81 additive action using equi-effective mixtures focusing on mortality, growth, and reproduction, and for
82 transcriptome signatures. *Daphnia* reproduce by cyclical parthenogenesis, thereby allowing the molecular
83 responses between compounds to be measured without the confounding effects of genetic variation among
84 strains in their sensitivity and in their regulatory pathways. A causal relationship between metal exposure and
85 adverse effect outcomes on somatic growth, reproduction, and transcriptional responses has been suggested,
86 with some knowledge of the underlying molecular mechanisms.[27] The completion of the *D. magna* reference
87 transcriptome now enables global gene regulation profiling by RNA sequencing (RNA-seq).[28,29] We elucidate
88 and compare *de novo* co-regulatory gene networks of Al, In, and their mixture to explore shared or distinct
89 functional biological processes. By comparing evolutionary-conserved *a priori* known adverse response
90 pathways for Al, we explore the application of this approach to assess combined effects at transcriptional
91 level. Ultimately, we discuss the utility of transcriptional responses for a chemical read-across, and whether
92 transcriptional responses may be linked to the chronic adverse outcomes, such as reproduction and growth.

93

94 **Materials and Methods**

Metals. Aluminum sulfate ($Al_2(SO_4)_3$), 99.99% trace metals basis, catalog no. 202614) and indium (III) chloride ($InCl_3$; anhydrous, 99.999% trace metals basis, catalog no. 429414) were obtained from Sigma Aldrich (Gillingham, UK).

Cultivation of *Daphnia magna* and Experimental Design. We used a *D. magna* genotype with inherited alleles from parents with different phenotypic and environmental backgrounds, such as the Xinb3 and Iinb1 genotype. Genotyping, breeding, and cultivation are described in the Supporting Information (SI).

During acute exposures, *Daphnia* neonates were kept at a density of ten organisms per 200 mL in artificial *Daphnia* medium (ADaM) without feeding. For each treatment group, four biological replicates were set up. At higher concentrations, $Al_2(SO_4)_3$ led to lowering of the pH due to the production of H_2SO_4. To maintain a stable pH during exposure, pH was adjusted using sodium hydroxide before starting the exposures to In and Al and mixtures.

Acute mixture experiments were designed according to an equi-effective protocol, where two metals were combined at concentrations producing equal mortality. This allows the comparison of responses of the single metals with those of equi-effective mixtures. Effect concentrations (ECs) were based on concentration-related 48 h mortality curves with single metals (4 replicates, n = 10).

Equi-effective concentrations of both metals were applied in mixtures to determine the activity of $EC_{0.625}$, $EC_{1.25}$, $EC_{2.5}$, EC_5, EC_{10}, EC_{20}, EC_{40}, EC_{80} mixtures. The effects were analyzed according to the CA concept, and based on the assumption that, for example, the mixture of $EC_{5(compound\ A)}$ and $EC_{5(compound\ B)}$ would lead to an overall additive effect of 10% in the mixture. To determine the onset of mortality, it was recorded continuously over 48 h. More than 80% of *Daphnia* survived the first 10 h in the EC_5 + EC_5 mixture and consequently, transcriptional effects of an equi-effective mixture of EC_5 + EC_5 and the single metal concentration of EC_{10} were assessed at this time-point. Following exposure, surviving *Daphnia* were immediately snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

Chronic exposure experiments were conducted using a 5-, 10-, and 20-fold dilution concentration of the 48 h 10% effect concentrations (EC_{10}) of the single metals (6.062, 3.031, 1.516 mg L^{-1} Al, 9.168, 4.584, 2.292 mg L^{-1} In), and EC_5 + EC_5 of the metal mixture. *Daphnia* were exposed until the release of the third brood or 21 days

121 at maximum. One organism was kept in a volume of 60 mL ADaM in glass beakers under 16:8 h light:dark
122 photoperiod and fed daily. A total of ten replicates per concentration group was set up and refreshed every
123 third day.

124 **Inductively-Coupled Plasma Mass-Spectrometry (ICP-MS) Analyses.** Concentrations of the soluble fraction of
125 aluminum (^{26}Al) and indium (^{115}In) isotopes in exposure media were quantified by inductively-coupled-plasma
126 mass-spectrometry (ICP-MS; Agilent 7500cx, Switzerland) equipped with an Octopole Reaction System,
127 pressurized with an optimized helium flow of 5 mL min^{-1}. Medium samples were filtered through a 0.45 µm
128 membrane, acidified to 1 % HNO_3 before analysis. Rubidium was used as internal standard.

129 **Molting, Growth, and Reproduction.** Endpoints for chronic exposures were assessed according to established
130 methods and described in SI including their statistical analyses.

131 **Bio-imaging.** The Laser Ablation ICP-MS (LA-ICP-MS) technique was applied for bio-imaging the elemental
132 distribution within *Daphnia* as previously described[30] and outlined in the SI.

133 **RNA Extraction, Library Preparation, and Sequencing.** Transcriptome analysis using RNA-seq was performed
134 of neonates exposed for 10 h to EC_{10} for Al or In, respectively, and EC5 for Al and In in the mixture exposure.
135 RNA of 20 exposed neonates of each of the four replicates was extracted using the RNeasy Mini Kit (Qiagen)
136 following the manufacturer's instructions including RNase-free DNase I treatment. *Daphnia* were homogenized
137 using the 2010 Geno/Grinder (SPEX SamplePrep, UK; 1750 rpm for 10 s). RNA quantity was measured using a
138 Nanodrop 8000 (Thermo Scientific, US), and integrity verified on a 2200 TapeStation system (Agilent
139 Technologies, US). Only samples with an RNA integrity number (RIN) higher than 7 were used for further
140 analysis. Poly(A)+ RNA was enriched using a NEBNext Poly(A) mRNA Magnetic Isolation Module. After reverse
141 transcription, a complementary cDNA library was constructed using the NEBnext Ultra Directional RNA Library
142 Prep Kit (New England Biolabs, U.S.).

143 Briefly, mRNA was further purified by exploiting the poly-A tails using NEBNext Oligo d(T) beads. Then, mRNA
144 was fragmented to suitable lengths, which were subsequently converted into cDNA by reverse transcriptase.
145 The fragmented cDNA was purified using AMPure XP beads, bound by oligonucleotide adaptor, followed by
146 PCR library enrichment. After library production, QC was performed using the TapeStation system with a High-

Sensitivity D1000 tape, confirming the size of the library. Quantitation was performed using the Kapa Library Quantitation Kit (Kapa Biosystems Ltd, UK) for Illumina Platforms, and equal molar quantities of each library mixed to produce a pooled library sample, which was tested again by the same procedures. A 2 nM pooled library sample was denatured using NaOH (per Illumina protocols), and loaded onto a Rapid-Run v2 slide using the Illumina cBot instrument at a 12 pM concentration. The cDNA was sequenced in paired-end sequencing-mode with 50 bp read length on an Illumina HiSeq 2500 machine using a v2 Rapid-Run SBS kit.

Bioinformatic analysis of RNA-seq data. A detailed description of the analysis is given in the SI. Briefly, several quality check steps were performed before resulting high-quality reads were mapped to the *D. magna de novo* transcriptome.[29] The transcript counts (number of mapped reads per transcript per sample) were summarized using Bioconductor package tximport (v1.6.0)[31] then normalized. We used DESeq2[32] to conduct differential gene expression analysis. Genes were considered differentially expressed (DE) if the resultant adjusted *p*-value < 0.1 (False discovery rate, FDR = 10%) for the purpose of reducing a Type II error (to falsely infer that there is no overlap of genes across treatments), and for discovering shared co-responsive gene networks in *Daphnia*'s response of the three treatments. We annotated DE gene sets based on their responses to each of the three trivalent metal treatments. Class-1 genes were differentially expressed in only 1/3 of the treatments. Class-2 genes were differentially expressed in 2/3 of the treatments. Class-3 genes were differentially expressed for Al, In and their mixture (3/3 of the treatments). An *ab initio* search for enriched gene sets among the treatments and shared co-regulated gene networks was also conducted.

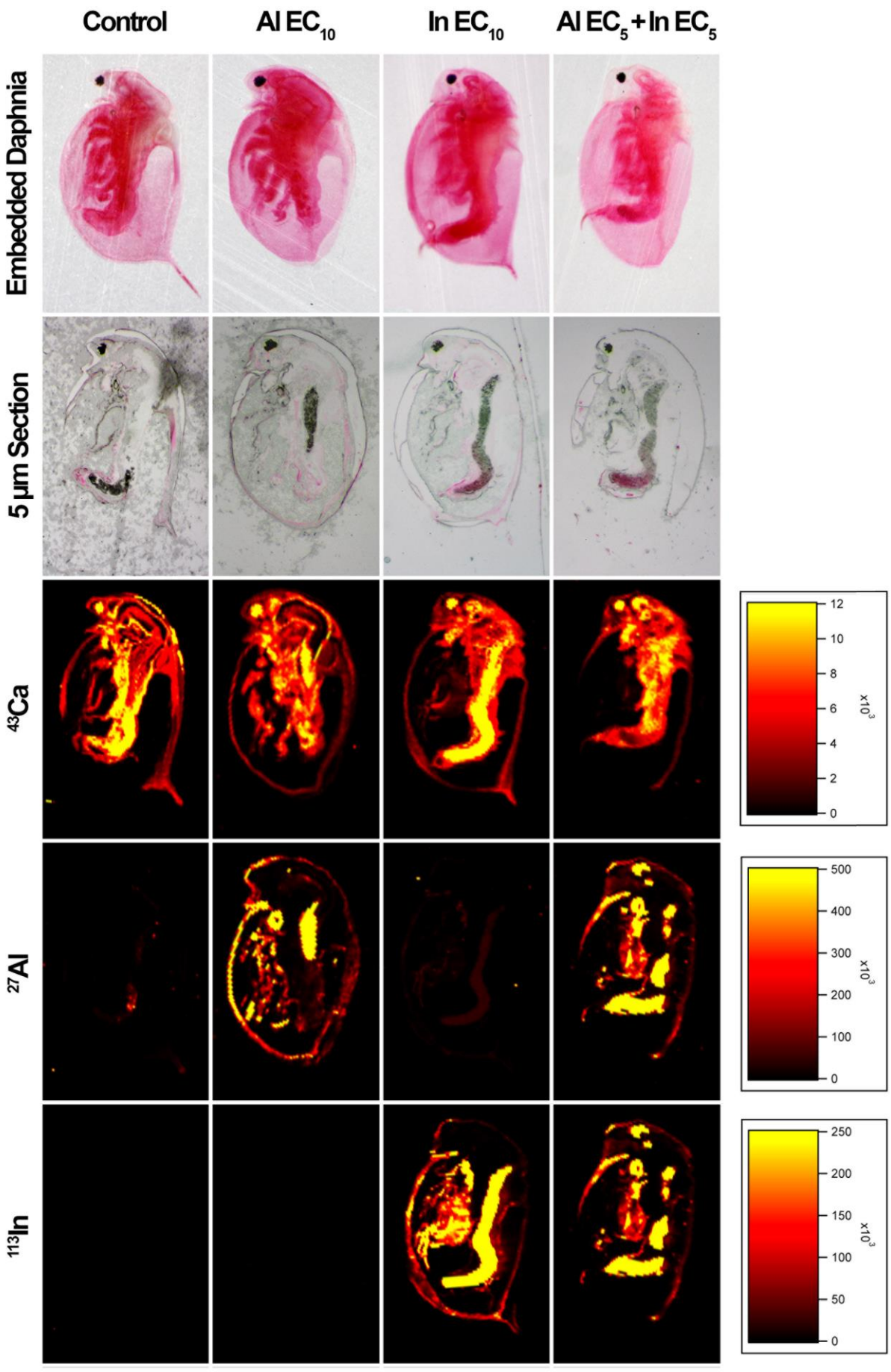
The responding genes were functionally analysed by a targeted approach using gene set enrichment analysis (GSEA) for an *a priori* gene set containing genes that are members of known pathways associated with Al exposure. Thus, the CA concept was tested by testing for additivity in similar gene sets for both compounds. This gene set was composed of all the known *D. magna* homologs to genes of the following seven pathways[33–36] identified for *D. pulex* using the Panther database[37]: Oxidative stress response, apoptosis signaling pathway, hypoxia response via HIF activation, p53 pathway, p53 pathway via glucose deprivation, p53 pathway feedback loop 1, p53 pathway feedback loop 2. We tested whether the genes from these pathways were enriching the top-ranked genes for all four conditions (Al *versus* control, In *versus* control, Al + In *versus* control, Al *versus* In). based on expression levels across all conditions. *Daphnia pulex* genes that belong to candidate general stress response pathways were retrieved from PROWLER[38] and mapped to their respective *D. magna* orthologues

using a custom python script and OrthoDB[39]. Gene differential expression matrices from DESeq2 were pre-ranked based on average LFC in gene expression. GSEA was then conducted using the GSEA tool (v3.0) of the candidate pathway genes against the pre-ranked gene list, using default [40,41] Reports containing enrichment scores, normalised enrichment scores and FDR for each analysis were generated, highlighting enrichment of genes from candidate pathways within the pre-ranked gene lists.

Results

Exposure concentrations. The measured soluble fraction of the Al and In concentrations in the transcriptomic experiment was lower than nominal and was 0.635 mg L^{-1} and 11.535 mg L^{-1}, respectively. During the 10 h exposure, the concentrations fell between 8.7 % (Al) and 11.6 % (In). Details are given in the SI including values (Table S1, SI).

Metal uptake. The LA-ICP-MS profiles of embedded *Daphnia* showed the distribution of Al and In in the organisms after ten hours (Figure 1). Al as an essential trace element[42] showed accumulation mainly in the carapace, which is also observed in the mixture, but also in the hind gut and midgut, as well as in the thoracic limbs, carrying the filtering screens. In controls, only minor levels of Al and In occurred. In showed a similar, if not almost identical accumulation as Al in the carapace, thoracic limbs, and gut. The metal pattern is distinctly different from calcium (Figure 1) and phosphorus (Figure S1, SI) delineating the carapace and gut, but also eyes, and blind gut (caecum) in case of calcium. ^{113}In to ^{115}In intensity is displayed according to its isotope ratio of 22 with both isotopes revealing similar images and intensity, therefore interferences can be ruled out (Figure S1, SI).


Control
Al EC10
In EC10
Al EC5 + In EC5
Embedded Daphnia
5 µm Section
43Ca
27Al
113In
x10^3
12
10
8
6
4
2
0
500
400
300
200
100
0
250
200
150
100
50
0

195

196

197 **Figure 1.** Laser-ablation ICP-MS elemental mapping of calcium (Ca), aluminum (Al), and indium (In) in 6 days
198 old *Daphnia magna* after a ten-hour exposure to measured concentrations of 0.635 mg L^{-1} Al, 11.535 mg L^{-1} In
199 (EC_{10} for neonates) and the equi-effective mixture of Al and In (0.596 and 9.096 mg L^{-1}), First row: paraffin
200 embedded *Daphnia magna*; second row: 5 µm section; third to the fith row: LA-ICP-MS images of elements
201 indicated on the left. Colour bars show the intensity in counts per seconds. The variation in the profile of the
202 animals is a result of the dehydration, embedding, and slicing procedure and thus images cannot be used as
203 species identification.

204 **Effects on Survival - Acute Exposures.** The mortality dose-response curve produced a steep hill slope (Al:
205 9.838, In: 18.62) for both metals after 24 h with nominal EC_{50} values of 44.27 mg L^{-1} (Al) and 58.93 mg L^{-1} (In;
206 Figure 2). The effects of equi-effective mixtures were greater than additive, as the curves were shifted left to
207 the predicted CA curve. Even a low concentration of EC_5 + EC_5 lead to about 70% mortality (Figure S2, SI). After
208 48 h, the EC_{50} value for Al and In was 35.91 mg L^{-1} and 54.49 mg L^{-1}, respectively, with a hill slope of 12.73 for
209 Al and 12.62 for In. At both exposure times, the EC_5 and EC_{10} values were relatively close to each other due to
210 the steep hill slope. Dose-response curves at 48 h were then taken as a basis to define nominal equi-effective
211 concentrations to be used for mixture experiments. Due to high mortality at 48 h, no comparison of single
212 metals and mixture exposures was possible. Mortality started to increase after ten hours for both metals at
213 lower concentrations, and even earlier (6 h) at higher concentrations (Figure S2, SI). The mixture of EC_5 + EC_5
214 induced an average mortality rate of 10% after ten hours. Consequently, for subsequent transcription analysis,
215 a ten-hour exposure and a combination of EC_5 + EC_5 and EC_{10} were chosen. All nominal EC values are listed in
216 Table S2 in the SI.

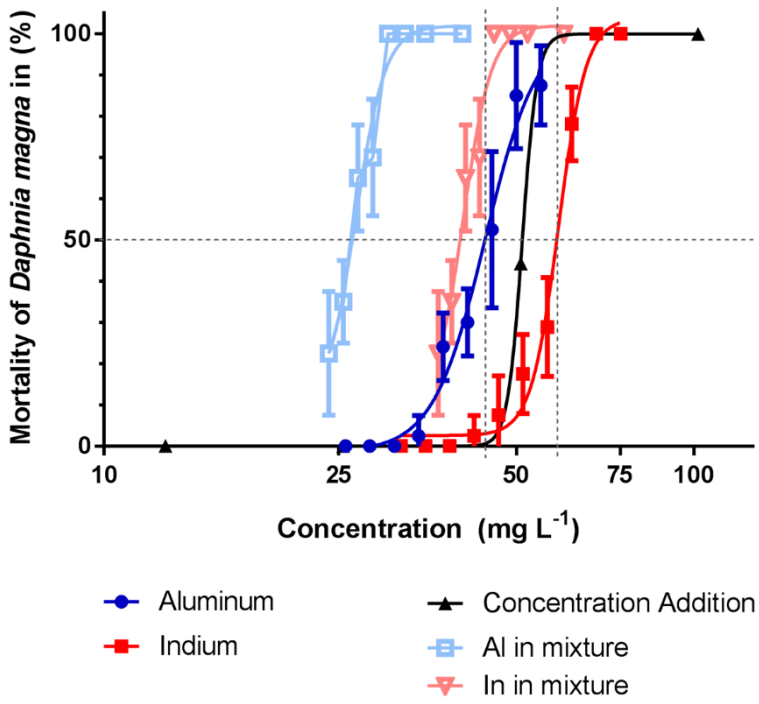


Figure 2. Mortality of *Daphnia magna* neonates exposed for 24 h to aluminum ($Al_2(SO_4)_3$) or indium ($InCl_3$) illustrating synergistic activity in the mixture. Hill slope for aluminum and indium is 9.838 and 18.62, respectively. For the subsequent transcriptomic experiments, a mixture exposure of EC5 + EC5 for 10 h was chosen allowing for survival of on average 90% of *Daphnia magna* (for time course study from 0 to 48 h see Figure S2, SI). Error bars represent the standard deviation (SD) of each exposure group (four replicates, n = 10).

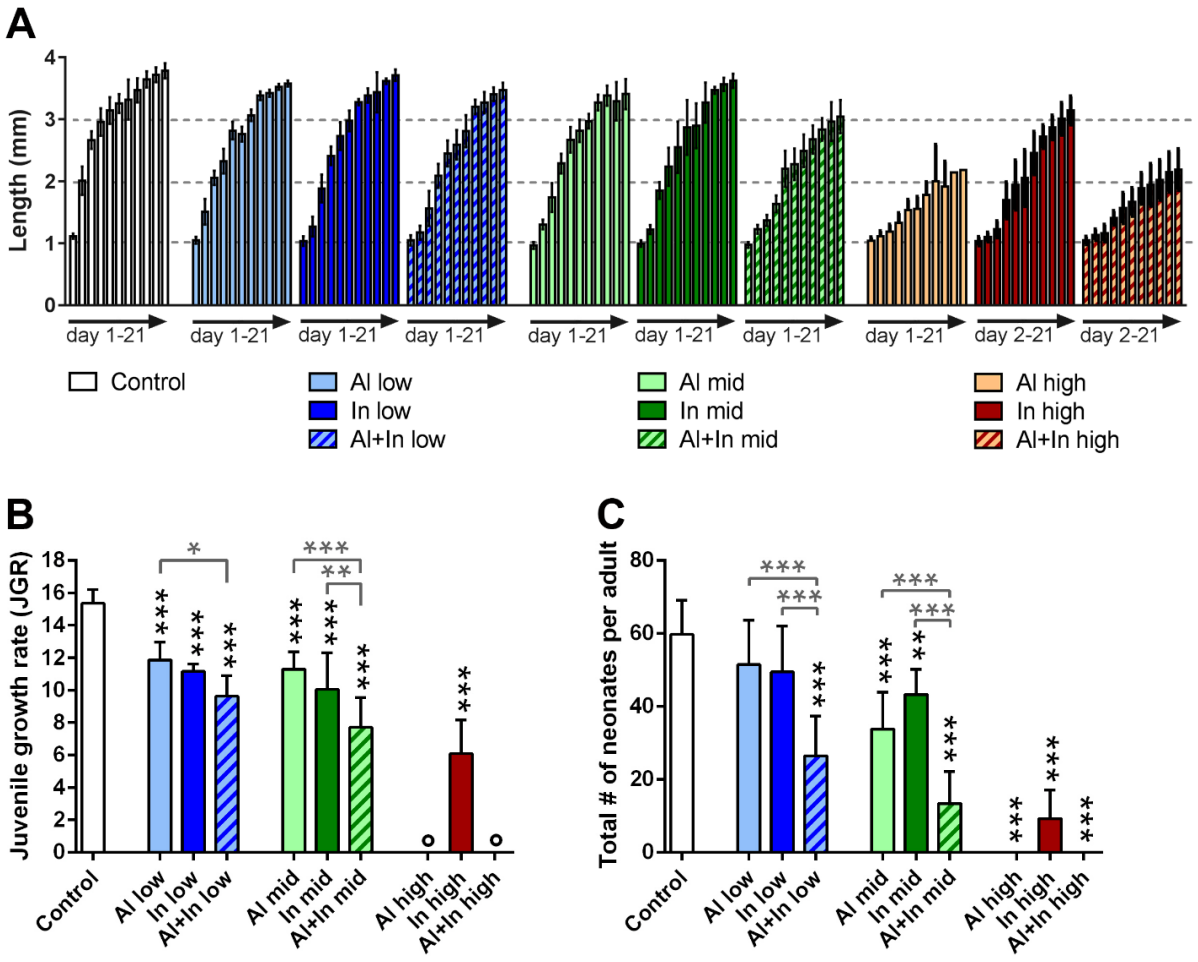
Effects on molting, growth, and reproduction – Chronic exposures. Concentrations used in chronic exposures were for Al 1.52 (low), 3.03 (mid), 6.06 (high) mg L^{-1}, for In 2.29 (low), 4.58 (mid), 9.17 mg L^{-1} (high), and for Al + In 1.43 + 2.17 (low), 2.85 + 4.33 (mid), 5.71 + 8.66 mg L^{-1} (high). Molting was recorded daily and plotted cumulative against time (Figure S3, SI). High Al concentration significantly decreased molting that did not appear in the binary mixture.

In the low concentration, both single metals and mixture led to a similar growth reduction in the first ten days. A strong reduction occurred in the middle concentration of the mixture, while in the highest concentration, growth was already minimal in the Al exposure after two days. Over all time points, exposed *Daphnia* showed a concentration-dependent decrease in growth (Figure 3A).

Age at maturity (Figure S4A, SI) was significantly delayed in all exposure groups (except in Al low) suggesting an effect on early life stages. This is even more evident when comparing juvenile growth rate (considering growth until sexual maturity; Figure 3B) with specific growth rate (considering growth until day 21; Figure S5A, SI). The juvenile growth rate was reduced in all concentrations and treatments, whereas the specific growth rate was reduced in the highest concentration only. The population growth rate was significantly reduced in all mid and high concentrations (Figure S5B, SI).

Mid and high concentrations of Al and In and all mixture concentrations decreased the total number of produced juveniles (Figure 3C). Effects on fecundity were higher in the mixtures than in single metal exposures and in high concentrations, any neonates were produced. Brood sizes increased from the first to the third brood in controls and single metals exposures (low and mid concentrations), whereas the high In and mid mixture concentration resulted in a decrease in the third brood size (Figure S4B, SI).

Al and In exhibited similar phenotypic responses in all the endpoints, except in population growth rate and age at maturity, where In induced a stronger effect than Al at low concentration, while Al had a stronger effect than In at high concentrations in molting and growth reduction.



246

247 **Figure 3.** Length, growth rate, and fecundity of *Daphnia magna* from day 1 (after 24 h of exposure) to 21
248 exposed to three concentrations of aluminum (Al), indium (In), and their equi-effective mixture concentration
249 (Al+In). (A) Carapace length over 21 days. Bars for day 1, 3, 5, 7, to 21 are shown. (B) Juvenile somatic growth
250 rates from day 2 to maturity. Circles indicate that, due to toxicity, none of the individuals in the group reached
251 maturity within 21 days, and therefore, were not included in statistical analysis. (C) Fecundity of *Daphnia* in
252 controls and exposure groups. The total number of neonates includes 3 brood releases or 21 experimental
253 days maximal. Mean ± SD. Black asterisks indicate significantly different to control, gray asterisks indicate the
254 difference between single compound and equi-effective mixture (n=10). Nominal concentrations are for Al
255 1.52 (low), 3.03 (mid), 6.06 (high) mg L^{-1}, for In 2.29 (low), 4.58 (mid), 9.17 mg L^{-1} (high), and for Al + In 1.43 +
256 2.17 (low), 2.85 + 4.33 (mid), 5.71 + 8.66 mg L^{-1} (high).

257

Transcriptomic profiles of *D. magna at similar effective concentrations*. The genotype of the IXF1 *D. magna* clone used in our study is confirmed by PCA analysis of the genetic variation (Figure S6, SI). Normalized counts for each gene of every replicate and the statistically significant differentially expressed (DE) genes across the three treatments compared against the control are given in Table S3, SI, the volcano plot in Figure S7, SI, and the heat map in Figure S8, SI. An adjusted p-value of 0.1 was applied, to broaden the gene list at reducing a Type II error, more stringent adjusted p-value of 0.05 are also given in Table S3, SI. The functional annotation of this gene set, including gene family from OrthoDB, InterPro, and derived Gene Ontology, as well as more comprehensive annotation using the 'Panther database' is provided in Table S3, SI. The same table also lists the inferred orthologs of all of the *Daphnia magna* loci in *D. pulex*, *Drosophila*, *Danio rerio*, and *Mus musculus*, including the *Daphnia* orthologs to Al responding genes from the previously published zebrafish study[43].

From among all the 597 DE genes in at least one treatment, the two most prominent molecular functions are antioxidant activity (GO:0016209) followed by catalytic activity (GO:0003824; Figure S9, SI). Although only nine genes are DE for both metals at the exclusion of their mixture (Figure 4), shared molecular functions are found for the class-1 gene sets that are DE for either the Al or In treatments, including binding, glutamate receptor activity, ligand-gated ion channel activity, signal transducer activity and transferase activity, transferring glycosyl groups (Table S3, Table S4, SI). Our experiments revealed only 40 class-3 genes that are DE across all three treatments (Figure 4, Table S3, SI). Inferred orthologs for other model species can be found in Table S3 (SI).

Exposure to Al and In resulted in differential expression of 155 and 135 genes, respectively. Among the fraction of uniquely expressed genes in each of the two conditions (45% and 41%), Al down-regulates the majority of responding genes (28 up *versus* 42 down), while In upregulates the majority of respective responding genes (37 up *versus* 18 down). Among the 463 genes that are DE when *Daphnia* were exposed to the mixture, a substantially larger fraction of genes are uniquely altered (77%), which suggests distinct gene responses compared to those induced by each component of the mixture. Clustering of the treatment groups highlights two characteristics of their DE genes (Figure S10, SI): (i) the Al and In replicated treatments cluster together; (ii) the mixture treatments cluster independently of the Al and In treatments. Therefore, the transcriptomes of Al and In are more alike than the transcriptome of the mixture treatment. Functional analysis of transcripts according to biological process, revealed processes that are unique to the mixture such

as calcium-mediated signalling, carbohydrate transport, cholesterol metabolic process, DNA recombination, DNA repair, endocytosis, ectoderm development, glycogen metabolic process, phospholipid metabolic process, protein lipidation, protein phosphorylation, response to abiotic stimulus, segment specification, and translation (for full list see Table S4, SI).

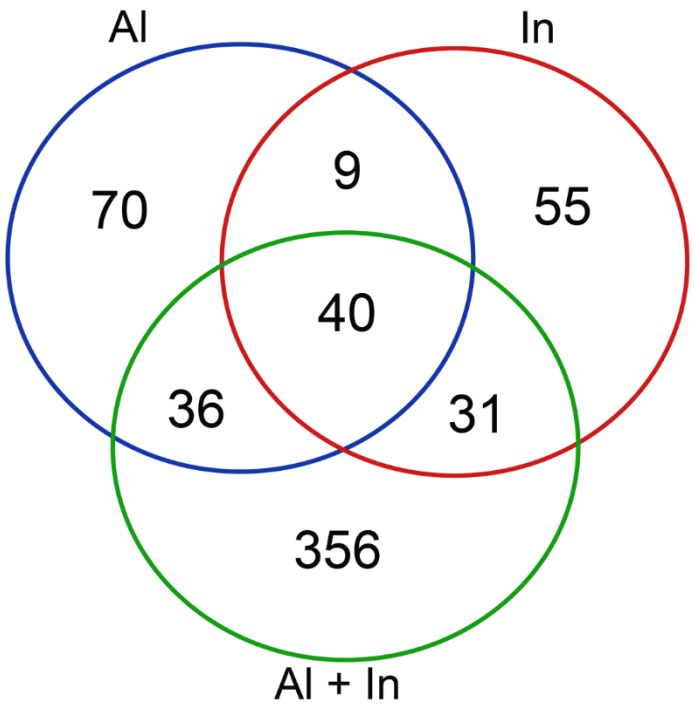


Figure 4. Venn diagram of all differentially expressed (DE) genes (p-adjusted < 0.1, n=4) determined by transcriptome sequencing of *Daphnia magna* exposed to measured concentrations of 0.635 mg L^{-1} aluminum (Al), 11.535 mg L^{-1} indium (In), and an equi-effective mixture of Al and In (0.596 and 9.096 mg L^{-1}), respectively, compared to non-exposed controls.

Only 49 DE genes are shared between Al and In from among 241 DE genes across both conditions and 40 of them are also DE in the mixture, which has an even larger number of responding genes at any *p*-adjusted threshold. Although the transcriptomes of Al and In exposure are more alike than that of the mixture, there

300 are few detectable functional classes of genes (biosynthetic process, catabolic process, cellular process,
301 nitrogen compound metabolic process) that are shared among all the treatment groups (Table S3, SI).

302 **Synergistic Equi-Effective Mixture Effects**. To explore potential synergistic effects, all class-3, class-2 and class-
303 1 DE genes were plotted comparing mixture *versus* single metal treatments (Figure 5). The ternary plot of
304 class-3 DE genes (Figure 5A) revealed six genes out of 40 (15%) with expression levels that are amplified by a
305 synergistic effect of Al and In as a mixture. These include a RH-associated glycoprotein (Dapma7bEVm004715)
306 that also functions as a transmembrane (cation) transporter, a sodium-independent sulphate anion
307 transmembrane transporter (Dapma7bEVm029187) with the annotated biological process of anion transport,
308 and a basic leucine zipper transcription factor (Dapma7bEVm005752) associated with many biological
309 processes including immune system processes. The remaining three genes have no known orthologs in other
310 model species (Table S3, SI).

311 The ternary plot of class-2 DE genes (Figure 5B) revealed 9 genes out of 76 (12%) with amplified
312 expression levels by interactions between the two metals. These include a gammy-butyrobetaine dioxygenase
313 (Dapma7bEVm002353), an extracellular matrix protein (Dapma7bEVm000277), a C1qdc1 protein
314 (Dapma7bEVm010318), and a small GTPase (Dapma7bEVm011784). For completeness, we identify 26 genes in
315 *Daphnia* that are most responsive to the mixture treatment (Figure 5C). Of these 41 genes, none are known
316 orthologs to Al or In responsive genes in other species. When the expected expression level of DE genes (by
317 the additive model) is regressed against the observed expression level under the equi-effective mixture
318 condition (Figure S11, SI), there is a statistically significant signal that the global effect of the mixture on gene
319 transcription is twice the predicted value under the additive CA model, irrespective of class-3 (r^2 value = 0.85)
320 or class-2 (r^2 value = 0.89) DE genes (Figure S11, SI). Thus, the annotated DE genes deviate from additivity.

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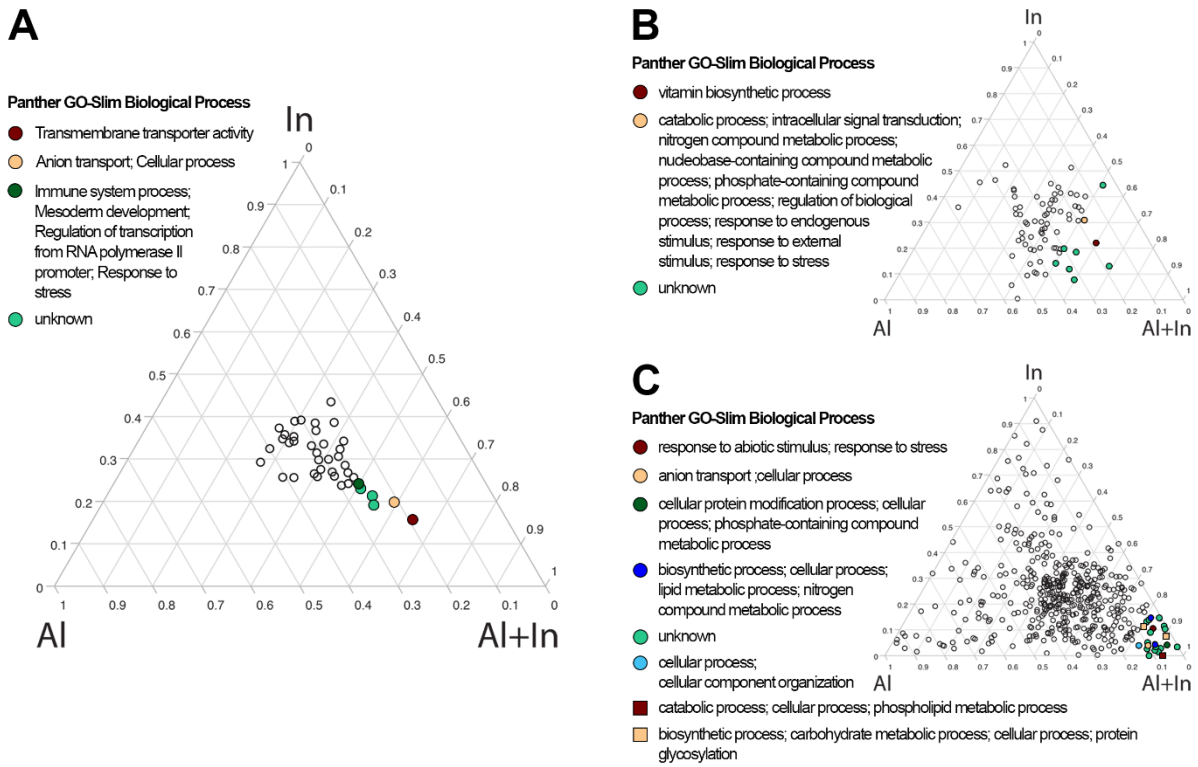

Figure 5. Comparison of response of all DE genes in the mixture exposure *versus* the two single metal treatments. Ternary plots depict differentially expressed (DE) genes for (A) all three conditions (class-3 DE genes), (B) two out of three conditions (class-2 DE genes), and (C) class-1 DE genes (n=4). The genes edging towards 'Al + In' correspond to the genes that have the largest residues and whose expression levels are most amplified in the mixture, whereas genes in the centre respond additively. The majority of genes respond independently in the mixture condition, as seen in (C). The functions of the genes are derived from Table S3 (SI) giving the residuals for each of the DE genes measured against the orthogonal regression lines in Figure S11 in the SI, which takes in account error estimates from both the expected and observed values.

Comparing Known Responsive Genes. The gene set enrichment analysis (GSEA) of stress pathways associated with Al exposures revealed that these *a priori* selected stress response genes from different organisms are also significantly *up-regulated* in *Daphnia*'s response to Al exposure (Figure 6A). By contrast, these stress response genes from within the same gene set significantly enrich the *down-regulated* genes in response to In exposure (Figure 6B), while in the mixture (Figure 6C), these genes enrich *both the up-regulated and down-regulated genes*. A direct comparison of Al *versus* In (Figure 6D) reinforces this finding of a near opposite response

between the two metals, by almost doubling the enrichment score for overexpressed Al genes, from 0.25 to >0.4. The mixture shows the same enrichment score as seen in the Al exposure (Figure S12, SI), indicating that a separate set of known gene responders are differentially expressed by exposure to Al and In present in the mixture, via either characteristic up-regulation or down-regulation associated with Al and In exposure respectively.

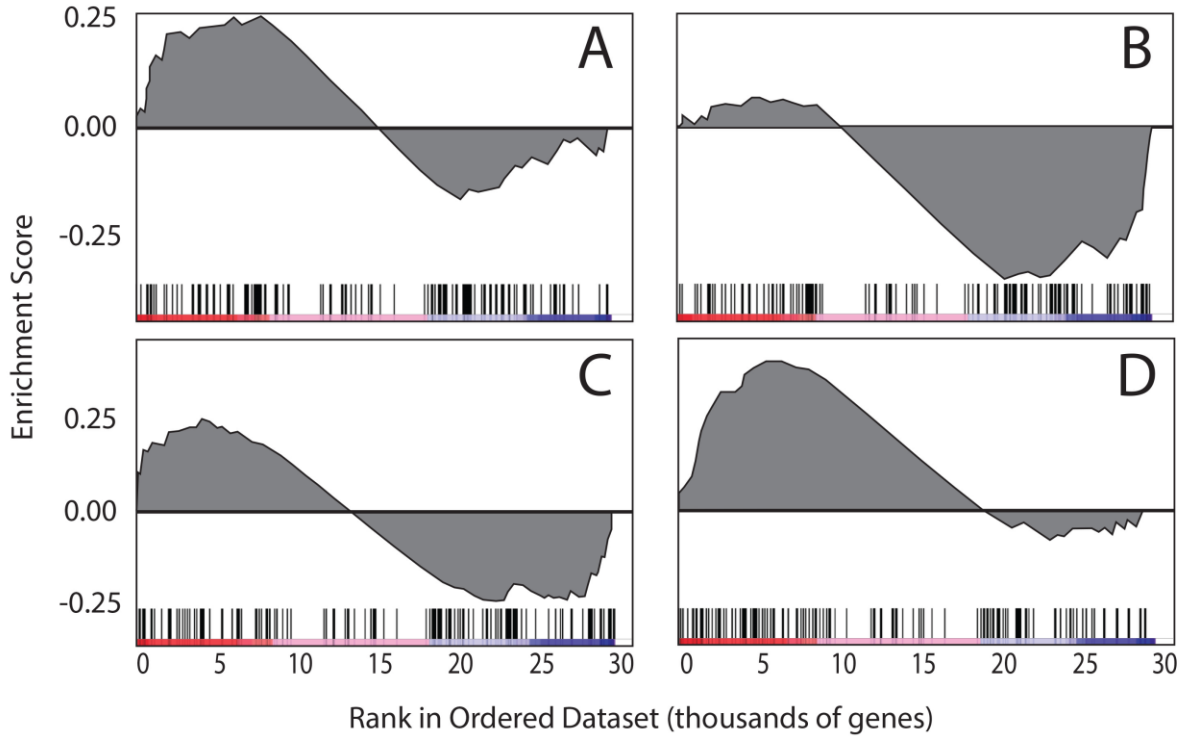

Figure 6: Gene set enrichment analysis (GSEA) of stress response pathways. GSEA on differentially expressed (DE) genes to (A) aluminum, (B) indium, (C) mixture, calculated through comparison *versus* control, or (D) between aluminum *versus* indium. *Daphnia magna* genes were aligned to their respective *D. pulex* orthologues and ordered based on their fold difference in expression. These genes were then analysed against *D. pulex* genes of candidate pathways retrieved from PANTHER. Candidate genes belonged to ontologies oxidative stress response, apoptosis signalling pathway, hypoxia response via HIF activation, p53 pathway, p53 pathway via glucose deprivation, p53 pathway feedback loop and p53 pathway feedback loop 2. Black bars form part of leading-edge analysis, highlighting genes that contribute most to enrichment scores. The coloured bars indicate the fold change in expression between compared datasets (red; increased expression, blue; decreased

expression). Enrichment score is an indication of how frequently candidate genes are found together (i.e. have similar expressional changes) in the rank-ordered dataset. Normalised enrichment scores (NES) and associated false discovery rate (FDR) are shown for each analysis with the exception of (C).

Comparing Unknown Responsive Genes. Untargeted differential expression analyses were employed in order to characterise putatively conserved unknown DE genes. Qualitative comparison of DE genes with the highest differences in treatment conditions (Figure S12, SI) highlights an overall disparity between each condition. Responses on individual DE genes vary substantially for all but a few genes, with no obvious similarity. Therefore, genes with the greatest differences in expression between the control and treatments show very little commonality in expression on individual gene level.

To test for potential conservation in DE genes, a network-based approach was employed, which groups similarly expressing genes in co-expression modules that can then be compared across metal treatments to identify unknown responders associated with novel expressional interactions. Networks pertaining to each metal treatment were compared using PCA (Figure S13, SI) based on the gene content of each module. Comparing three principle components highlights four distinct clusters of co-expressional modules across treatments; Module 1 (Al 30, In 14, AlIn 13), Module 2 (Al 14, In 3, AlIn 9), Module 3 (Al 4, In 32, AlIn 2), Module 4 (Al 23, In 24, AlIn 27). A majority of co-expression modules do not cluster independently, therefore the significance of these clusters is in the potential novel and conserved functionality served by the composite genes responding in similar manners across the treatments. Taken together, comparison of DE genes across treatments highlights low conservation in highest responding DE genes and at a transcriptional network level, with shared responses at a network level associating novel gene interactions with Al, In and mixture exposures.

DISCUSSION

In our study, we observe synergistic effects when combining two metals. By performing the first global transcriptome analysis in the ecologically relevant *Daphnia* on In and its mixture with Al we substantiate the notion that different target MoAs can potentiate the response. We provide a straightforward transcriptional

analysis of mixtures, and observe that the transcriptional data are paralleled with synergistic effects on growth and reproduction.

More than additive interaction was found in adult *Daphnia* on mortality and chronic toxicity, including reduced growth and fecundity. In neonates, the global transcription analysis revealed that the number of differentially expressed genes in the mixture clearly exceeded the number of each single metal treatment, which is in accordance with microarray studies describing transcriptional activity of mixtures.[18,44] Here, we took a novel approach using RNA-seq and compared the DE genes of the single metal exposures with their mixture, revealing distinct classes of genes that deviate from the expected additive effect of the two similarly acting compounds. Synergistically acting *de novo* co-regulatory genes were identified and assigned to biological processes such as immune system process, transmembrane transporter activity, and several metabolic processes, including lipid and carbohydrate metabolism and catabolic processes (Figure 5, Table S3, SI).

The disruption in metabolic processes likely mirror the organisms elevated need for energy coping with the stress situation. Disruption of energy homeostasis causing depleted lipid reserves is directly related to reduced growth in *Daphnia*,[45–47] which is a common adverse effect of metals.[48] In our study, growth reduction occurred already after two days resulting in a reduced juvenile growth rate and delayed age at maturity, both stronger affected in the equi-effective mixture. Consequently, the depleted energy reserves may impair reproduction. Especially reduced clutch size in the third brood is indicative for exhausted energy stores. However, the link of gene expression to organismal level responses is currently limited.[49]

In addition, depleted energy sources may also originate from reduced food intake.[50] Bioimaging showed that both metals were accumulated on the carapace and in the filtering screens, possibly physically hindering a constant flow of algae and thus reduce net energy and growth. Reduced ingestion rate in *Daphnia* after cadmium exposure was suggested to be a key mechanism impairing energy uptake and thus reproduction and growth.[51] Despite potentially reduced ingestion rate, the highest concentrations of Al and In were detected in the midgut, which is in line with other studies on metal uptake in *D. magna*.[52] Al crossing cell membranes by endocytosis are found accumulated in lysosomes.[52,53] The lysosome cellular component (Table S3, SI) was enriched in all the treatment groups and endocytosis in the mixture, suggesting their involvement

406 in Al and In accumulation and effects. Furthermore, Al may be transported by yet unknown transporters in
407 *Daphnia*, similar to animal transporters, or transporters of the ABC or MATE family.[54,55]

408 In our study, we demonstrate the utility of genome-wide transcriptional responses to assess
409 additivity. We propose the use of GSEA to test for enrichment of *a priori* defined set of genes across
410 treatments, whereby compounds that differ in their MoA also differ in their profiles. This requirement differs
411 from the more stringent use of informative molecular profiles as a basis for reporting chemical's MoA, where
412 additional evidence is needed to establish causation between chemicals and their adverse effects. The GSEA
413 result suggests that the gene set known to be involved in stress response pathways to Al respond as predicted
414 in *Daphnia*, yet the same gene set is expressing an opposite effect when *Daphnia* are exposed to In. The
415 response to In, as well as to the mixture, indicates that expressions of these same genes are affected in
416 disparate and putatively opposite manners between the two metals. The deviation from additivity in the
417 transcriptional alterations in mixtures is likely a complex gene regulation and evidence from toxicogenomic
418 studies pile up, showing that mixture expression profiles represent not merely the additive sum of individual
419 compounds fingerprint and converging pathways can be activated leading to a faster or stronger response.[18,56–
420 [58] Therefore, this straightforward analysis elucidating gene sets deviating from additivity can be a powerful
421 tool in the mixture assessment.

422 Chemical safety legislations are often relying on chemical structure-base 'read-across' and
423 'quantitative structure-activity relationship' (QSAR) predictions at the expense of reporting actual toxicity data.
424 In the read-across approach, toxicity information for one well-studied chemical is used to predict the same
425 toxicological endpoint for another, i.e. emerging, chemical by virtue of their structural similarity or on the basis
426 of shared molecular responses.[59] However, the level of certainty of these read-across and QSAR predictions is
427 unsatisfactorily low thereby undermining the purpose of chemical safety legislation. Our approach provides a
428 simple test for similarity in transcriptional fingerprints and thereby support chemical safety assessment.

429 Together, our study leads to the conclusion that Al and In mixtures have more than additive
430 phenotypic effects in equi-effective concentrations, which is paralleled by expressional alterations of
431 substantially more genes, and more importantly, by synergistically responding DE genes. Reduced growth and
432 reproduction may be related to altered genes in energy metabolism processes which has important ecological

implications. We show that genome-wide transcriptional fingerprints provide a tool for reasonably rapid assessment for additivity of two compounds.

ASSOCIATED CONTENT

Supporting Information includes the description of cultivation of *Daphnia magna*, experimental design, chronic toxicity data, statistical analysis of phenotypic responses, bioimaging using laser-ablation ICP-MS, as well as results of chemical analysis, effective concentrations of single compounds, and biological processes assigned to Al and In treatment as well as figures on laser-ablation ICP-MS elemental mapping of ^{115}In and ^{31}P, time-course study of single compounds, number of molts, clutch sizes and age at maturity, specific growth rate and population growth rate, PCA analysis of genetic variation, volcano plots, heat map of all transcripts, functional annotations of DE genes, MDS, regression analysis, top 100 ranked genes, and PCA of co-expressional modules. The XLSX table S3 contains information on normalized mapping, DESeq results, annotations, orthologs, and residuals. All Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org. The sequence data are available at the NCBI BioProject database under accession number PRJNA508640.

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REFERENCES

(1) Zimmermann, Y.-S.; Schäffer, A.; Hugi, C.; Fent, K.; Corvini, P. F.-X. F.-X.; Lenz, M. Organic photovoltaics: potential fate and effects in the environment. *Environ. Int.* **2012**, *49*, 128–140.

https://doi.org/10.1016/j.envint.2012.08.015.

(2) Gensemer, R. W.; Playle, R. C. The bioavailability and toxicity of aluminum in aquatic environments. *Crit. Rev. Environ. Sci. Technol.* **1999**, *29* (4), 315–450. https://doi.org/10.1080/10643389991259245.

(3) Boughriet, A.; Proix, N.; Billon, G.; Recourt, P.; Ouddane, B. Environmental impacts of heavy metal discharges from a smelter in Deûle-canal sediments (Northern France): Concentration levels and chemical fractionation. *Water Air Soil Pollut.* **2007**, *180* (1–4), 83–95. https://doi.org/10.1007/s11270-006-9252-5.

(4) Kortenkamp, A.; Faust, M. Regulate to reduce chemical mixture risk. *Science.* **2018**, *361* (6399), 224–226. https://doi.org/10.1126/science.aat9219.

(5) Cedergreen, N. Quantifying Synergy: A systematic review of mixture toxicity studies within environmental toxicology. *PLoS One* **2014**, *9* (5), e96580. https://doi.org/10.1371/journal.pone.0096580.

(6) Loewe, S.; Muischnek, H. Über Kombinationswirkungen. *Arch. Exp. Pathol. Pharmakol.1* **1926**, *114*, 313–326. https://doi.org/10.1007/BF01859944

(7) Altenburger, R.; Scholz, S.; Schmitt-Jansen, M.; Busch, W.; Escher, B. I. Mixture toxicity revisited from a toxicogenomic perspective. *Environ. Sci. Technol.* **2012**, *46* (5), 2508–2522. https://doi.org/10.1021/es2038036.

(8) Bliss, C. I. The Toxicity of Poisons Applied Jointly. *Ann. Appl. Biol.* **1939**, *26*, 585–615. https://doi/abs/10.1111/j.1744-7348.1939.tb06990.x

(9) Nys, C.; Versieren, L.; Cordery, K.; Blust, R.; Smolders, E.; De Schamphelaere, K. A. C. A Systematic evaluation of chronic metal mixture toxicity to three species and implications for risk assessment. *Environ. Sci. Technol.* **2017**, *51*, 4615−4623. https://doi.org/10.1021/acs.est.6b05688.

(10) Kortenkamp, A.; Backhaus, T.; Faust, M. State of the art report on mixture toxicity. Final report to the European Commission.

http://ec.europa.eu/environment/chemicals/effects/pdf/report_mixture_toxicity.pdf (accessed Aug 27, 2018).

(11) Vandenbrouck, T.; Jones, O. a H.; Dom, N.; Griffin, J. L.; De Coen, W. Mixtures of similarly acting compounds in *Daphnia magna*: From gene to metabolite and beyond. *Environ. Int.* **2010**, *36* (3), 254–268. https://doi.org/10.1016/j.envint.2009.12.006.

(12) Christen, V.; Crettaz, P.; Fent, K. Additive and synergistic antiandrogenic activities of mixtures of azol fungicides and vinclozolin. *Toxicol. Appl. Pharmacol.* **2014**, *15* (3), 455–466. https://doi.org/10.1016/j.taap.2014.06.025.

(13) Christen, V.; Crettaz, P.; Oberli-Schrämmli, A.; Fent, K. Antiandrogenic activity of phthalate mixtures: Validity of concentration addition. *Toxicol. Appl. Pharmacol.* **2012**, *259* (2), 169–176. https://doi.org/10.1016/j.taap.2011.12.021.

(14) Kunz, P. Y.; Fent, K. Estrogenic activity of ternary UV filter mixtures in fish (*Pimephales Promelas*) - An analysis with nonlinear isobolograms. *Toxicol. Appl. Pharmacol.* **2009**, *234* (1), 77–88. https://doi.org/10.1016/j.taap.2008.09.032.

(15) Thrupp, T. J.; Runnalls, T. J.; Scholze, M.; Kugathas, S.; Kortenkamp, A.; Sumpter, J. P. The consequences of exposure to mixtures of chemicals: Something from 'nothing' and 'a lot from a little' when fish are exposed to steroid hormones. *Sci. Total Environ.* **2018**, *619–620*, 1482–1492. https://doi.org/10.1016/J.SCITOTENV.2017.11.081.

(16) Wang, D.; Hu, J.; Forthaus, B. E.; Wang, J. Synergistic toxic effect of nano-Al_2O_3 and As(V) on *Ceriodaphnia dubia*. *Environ. Pollut.* **2011**, *159* (10), 3003–3008. https://doi.org/10.1016/j.envpol.2011.04.019.

(17) Nys, C.; Asselman, J.; Hochmuth, J. D.; Janssen, C. R.; Blust, R.; Smolders, E.; De Schamphelaere, K. a. C. Mixture toxicity of nickel and zinc to *Daphnia magna* Is noninteractive at low effect sizes but becomes synergistic at high effect sizes. *Environ. Toxicol. Chem.* **2015**, *34* (5), 1091–1102. https://doi.org/10.1002/etc.2902.

(18) Vandenbrouck, T.; Soetaert, A.; van der Ven, K.; Blust, R.; De Coen, W. Nickel and binary metal mixture responses in *Daphnia magna*: Molecular fingerprints and (sub)organismal effects. *Aquat. Toxicol.* **2009**, *92* (1), 18–29. https://doi.org/10.1016/j.aquatox.2008.12.012.

(19) White, S. J. O.; Hemond, H. F. The Anthrobiogeochemical cycle of indium: A review of the natural and anthropogenic cycling of indium in the environment. *Crit. Rev. Environ. Sci. Technol.* **2012**, *42* (2), 155–186. https://doi.org/10.1080/10643389.2010.498755.

(20) Warby, R. a F.; Johnson, C. E.; Driscoll, C. T. Changes in aluminum concentrations and speciation in lakes across the Northeastern U.S. following reductions in acidic deposition. *Environ. Sci. Technol.* **2008**, *42* (23), 8668–8674. https://doi.org/10.1021/es801125d.

(21) Mujika, J. I.; Rezabal, E.; Mercero, J. M.; Ruipérez, F.; Costa, D.; Ugalde, J. M.; Lopez, X. Aluminium in biological environments: A computational approach. *Comput. Struct. Biotechnol. J.* **2014**, *9* (15), e201403002. https://doi.org/10.5936/CSBJ.201403002.

(22) Lukiw, W. J.; Percy, M. E.; Kruck, T. P. Nanomolar aluminum induces pro-inflammatory and pro-apoptotic gene expression in human brain cells in primary culture. *J. Inorg. Biochem.* **2005**, *99* (9), 1895–1898. https://doi.org/10.1016/j.jinorgbio.2005.04.021.

(23) Guo, C.-H.; Chen, P.-C.; Hsia, S.; Hsu, G.-S. W.; Liu, P.-J. The relationship of plasma aluminum to oxidant–antioxidant and inflammation status in asthma patients. *Environ. Toxicol. Pharmacol.* **2013**, *35* (1), 30–38. https://doi.org/10.1016/j.etap.2012.10.005.

(24) Rizvi, S. H. M.; Parveen, A.; Verma, A. K.; Ahmad, I.; Arshad, M.; Mahdi, A. A. Aluminium induced endoplasmic reticulum stress mediated cell death in SH-SY5Y neuroblastoma cell line is independent of p53. *PLoS One* **2014**, *9* (5), e98409. https://doi.org/10.1371/journal.pone.0098409.

(25) Brun, N. R.; Christen, V.; Furrer, G.; Fent, K. Indium and indium tin oxide induce endoplasmic reticulum stress and oxidative stress in zebrafish (*Danio rerio*). *Environ. Sci. Technol.* **2014**, *48*, 11679–11687.

(26) Zurita, J. L.; Jos, A.; del Peso, A.; Salguero, M.; Cameán, A. M.; López-Artíguez, M.; Repetto, G. Toxicological assessment of indium nitrate on aquatic organisms and investigation of the effects on the

PLHC-1 fish cell line. *Sci. Total Environ.* **2007**, *387* (1–3), 155–165. https://doi.org/10.1016/j.scitotenv.2007.07.057.

(27) Poynton, H. C.; Varshavsky, J. R.; Chang, B.; Cavigiolio, G.; Chan, S.; Holman, P. S.; Loguinov, A. V; Bauer, D. J.; Komachi, K.; Theil, E. C.; Perkins, E. J.; Hughes, O.; Vulpe, C. D. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. *Environ. Sci. Technol.* **2007**, *41* (3), 1044–1050. https://doi.org/10.1021/ES0615573

(28) Daphnia Genome Consortium. Daphnia magna genome sequence project.

(29) Orsini, L.; Gilbert, D.; Podicheti, R.; Jansen, M.; Brown, J. B.; Solari, O. S.; Spanier, K. I.; Colbourne, J. K.; Rush, D.; Decaestecker, E.; Asselman, J.; De Schamphelaere, K. A. C.; Ebert, D.; Haag, C. R.; Kvist, Jouni; Laforsch, C.; Petrusek, A.; Beckerman, A. P.; Little, T. J.; Chaturvedi, A.; Pfrender, M. E.; De Meester, L.; Frilander, M. J. *Daphnia magna* transcriptome by RNA-seq across 12 environmental stressors. *Sci. Data* **2016**, *3*, 160030. https://doi.org/10.1038/sdata.2016.30.

(30) Brun, N. R.; Lenz, M.; Wehrli, B.; Fent, K. Comparative effects of zinc oxide nanoparticles and dissolved zinc on zebrafish embryos and eleuthero-embryos: Importance of zinc ions. *Sci. Total Environ.* **2014**, *476–477*, 657–666. https://doi.org/10.1016/j.scitotenv.2014.01.053.

(31) Soneson, C.; Love, M. I.; Robinson, M. D. Differential analyses for RNA-seq: Transcript-level estimates improve gene-level inferences. *F1000Research* **2015**, *4*, 1521. https://doi.org/10.12688/f1000research.7563.2.

(32) Love, M. I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15* (12), 550. https://doi.org/10.1186/s13059-014-0550-8.

(33) Bustamante, J.; Dock, L.; Vahter, M.; Fowler, B.; Orrenius, S. The semiconductor elements arsenic and indium induce apoptosis in rat thymocytes. *Toxicology* **1997**, *118* (2–3), 129–136. https://doi.org/10.1016/S0300-483X(96)03607-4.

(34) Mailloux, R. J.; Lemire, J.; Appanna, V. D. Hepatic response to aluminum toxicity: Dyslipidemia and liver diseases. *Exp. Cell Res.* **2011**, *317* (16), 2231–2238. https://doi.org/10.1016/J.YEXCR.2011.07.009.

(35) Kawahara, M.; Kato-Negishi, M. Link between aluminum and the pathogenesis of Alzheimer's disease: The integration of the aluminum and amyloid cascade hypotheses. *Int. J. Alzheimers. Dis.* **2011**. https://doi.org/10.4061/2011/276393.

(36) Badding, M. A.; Stefaniak, A. B.; Fix, N. R.; Cummings, K. J.; Leonard, S. S. Cytotoxicity and characterization of particles collected from an indium-tin oxide production facility. *J. Toxicol. Environ. Health. A* **2014**, *77* (20), 1193–1209. https://doi.org/10.1080/15287394.2014.920757.

(37) Mi, H.; Dong, Q.; Muruganujan, A.; Gaudet, P.; Lewis, S.; Thomas, P. D. PANTHER Version 7: Improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. *Nucleic Acids Res.* **2010**, *38*, D204–D210. https://doi.org/10.1093/nar/gkp1019.

(38) Huber, W.; Carey, V. J.; Gentleman, R.; Anders, S.; Carlson, M.; Carvalho, B. S.; Bravo, H. C.; Davis, S.; Gatto, L.; Girke, T.; Gottardo, R.; Hahne, F.; Hansen, K.D.; Irizarry, R.A.; Lawrence, M.; Love, M.I.; MacDonald, J.; Obenchain, V.; Oleś, A.K.; Pagès, H.; Reyes, A.; Shannon, P.; Smyth, G.K.; Tenenbaum, D.; Waldron, L.; Morgan, M. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat. Methods* **2015**, *12* (2), 115–121. https://doi.org/10.1038/nmeth.3252.

(39) Zdobnov, E. M.; Tegenfeldt, F.; Kuznetsov, D.; Waterhouse, R. M.; Simão, F. A.; Ioannidis, P.; Seppey, M.; Loetscher, A.; Kriventseva, E. V. OrthoDB v9.1: Cataloging evolutionary and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs. *Nucleic Acids Res.* **2017**, *45* (D1), D744–D749. https://doi.org/10.1093/nar/gkw1119.

(40) Mootha, V. K.; Lindgren, C. M.; Eriksson, K.-F.; Subramanian, A.; Sihag, S.; Lehar, J.; Puigserver, P.; Carlsson, E.; Ridderstråle, M.; Laurila, E.; Houstis, N.; Daly, M.J.; Patterson, N.; Mesirov, J.P.; Golub, T.R.; Tamayo, P.; Spiegelman, B.; Lander, E.S.; Hirschhorn, J.N.; Altshuler, D.; Groop, L.C. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* **2003**, *34* (3), 267–273. https://doi.org/10.1038/ng1180.

(41) Subramanian, A.; Tamayo, P.; Mootha, V. K.; Mukherjee, S.; Ebert, B. L.; Gillette, M. A.; Paulovich, A.; Pomeroy, S. L.; Golub, T. R.; Lander, E. S.; Mesirov, J.P. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* **2005**,

583 *102* (43), 15545–15550. https://doi.org/10.1073/pnas.0506580102.

584 (42) Quiroz-Vázquez, P.; Sigee, D. C.; White, K. N. Bioavailability and toxicity of aluminium in a model
585 planktonic food chain (*Chlamydomonas–Daphnia*) at neutral pH. *Limnol. - Ecol. Manag. Inl. Waters*
586 **2010**, *40* (3), 269–277. https://doi.org/10.1016/j.limno.2009.10.007.

587 (43) Griffitt, R. J.; Feswick, A.; Weil, R.; Hyndman, K.; Carpinone, P.; Powers, K.; Denslow, N. D.; Barber, D. S.
588 Investigation of acute nanoparticulate aluminum toxicity in zebrafish. *Environ. Toxicol.* **2011**, *26*, 541–
589 551. https://doi.org/10.1002/tox.

590 (44) Finne, E. F.; Cooper, G. a.; Koop, B. F.; Hylland, K.; Tollefsen, K. E. Toxicogenomic responses in rainbow
591 trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals and a synthetic mixture. *Aquat.*
592 *Toxicol.* **2007**, *81*, 293–303. https://doi.org/10.1016/j.aquatox.2006.12.010.

593 (45) Li, M.; Czymmek, K. J.; Huang, C. P. Responses of *Ceriodaphnia dubia* to TiO_2 and Al_2O_3 nanoparticles: A
594 dynamic nano-toxicity assessment of energy budget distribution. *J. Hazard. Mater.* **2011**, *187* (1–3),
595 502–508. https://doi.org/10.1016/j.jhazmat.2011.01.061.

596 (46) De Coen, W. M.; Janssen, C. R. The missing biomarker link: Relationships between effects on the
597 cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding
598 population characteristics. *Environ. Toxicol. Chem.* **2003**, *22* (7), 1632–1641.
599 https://doi.org/10.1002/etc.5620220727.

600 (47) Soetaert, A.; van der Ven, K.; Moens, L. N.; Vandenbrouck, T.; van Remortel, P.; De Coen, W. M.
601 *Daphnia magna* and ecotoxicogenomics : Gene expression profiles of the anti-ecdysteroidal fungicide
602 fenarimol using energy-, molting- and life stage-related cDNA libraries. *Chemosphere* **2007**, *67* (1), 60–
603 71. https://doi.org/10.1016/j.chemosphere.2006.09.076.

604 (48) De Schamphelaere, K. a C.; Canli, M.; Van Lierde, V.; Forrez, I.; Vanhaecke, F.; Janssen, C. R.
605 Reproductive toxicity of dietary zinc to *Daphnia magna*. *Aquat. Toxicol.* **2004**, *70* (3), 233–244.
606 https://doi.org/10.1016/j.aquatox.2004.09.008.

607 (49) De Coninck, D. I. M.; Asselman, J.; Glaholt, S.; Janssen, C. R.; Colbourne, J. K.; Shaw, J. R.; De

Schamphelaere, K. A. C. Genome-wide transcription profiles reveal genotype-dependent responses of biological pathways and gene-families in *Daphnia* exposed to single and mixed stressors. *Environ. Sci. Technol.* **2014**, *48*, 3513–3522. https://doi.org/10.1021/es4053363.

(50) Gorokhova, E.; Rivetti, C.; Furuhagen, S.; Edlund, A.; Ek, K.; Breitholtz, M. Bacteria-mediated effects of antibiotics on *Daphnia* nutrition. *Environ. Sci. Technol.* **2015**, *49* (9), 5779–5787. https://doi.org/10.1021/acs.est.5b00833.

(51) Guan, R.; Wang, W. X. Cd and Zn uptake kinetics in *Daphnia magna* in relation to Cd exposure history. *Environ. Sci. Technol.* **2004**, *38* (22), 6051–6058. https://doi.org/10.1021/es049562z.

(52) Marwa, M.; Adrian, F.; Nedra, B.; Samira, M.; Horea, M.; Walid-Habib, T.; Baati, R.; Leila, T. The role of lysosomes in the phenomenon of concentration of aluminum and indium in the female reproductive system. An ultrastructural study. *J. Trace Elem. Med. Biol.* **2017**, *44* (February), 59–64. https://doi.org/10.1016/j.jtemb.2017.05.009.

(53) Exley, C.; Mold, M. J. The Binding, transport and fate of aluminium in biological cells. *J. Trace Elem. Med. Biol.* **2015**, *30*, 90–95. https://doi.org/10.1016/j.jtemb.2014.11.002.

(54) Miyauchi, H.; Moriyama, S.; Kusakizako, T.; Kumazaki, K.; Nakane, T.; Yamashita, K.; Hirata, K.; Dohmae, N.; Nishizawa, T.; Ito, K.; Miyaji, T.; Moriyama, Y.; Ishitani, R.; Nureki, O. Structural basis for xenobiotic extrusion by eukaryotic MATE transporter. *Nat. Commun.* **2017**, *8*, 1-11. https://doi.org/10.1038/s41467-017-01541-0.

(55) Liu, J.; Piñeros, M. A.; Kochian, L. V. The role of aluminum sensing and signaling in plant aluminum resistance. *J. Integr. Plant Biol.* **2014**, *56* (3), 221–230. https://doi.org/10.1111/jipb.12162.

(56) Zucchi, S.; Mirbahai, L.; Castiglioni, S.; Fent, K. Transcriptional and physiological responses induced by binary mixtures of drospirenone and progesterone in zebrafish (*Danio rerio*). *Environ. Sci. Technol.* **2014**, *48*, 3523–3531.

(57) Rossier, N. M.; Chew, G.; Zhang, K.; Riva, F.; Fent, K. Activity of binary mixtures of drospirenone with progesterone and 17α-ethinylestradiol *in vitro* and *in vivo*. *Aquat. Toxicol.* **2016**, *174*, 109–122.

https://doi.org/10.1016/j.aquatox.2016.02.005.

(58) Siegenthaler, P. F.; Bain, P.; Riva, F.; Fent, K. Effects of antiandrogenic progestins, chlormadinone and cyproterone acetate, and the estrogen 17α-ethinylestradiol (EE2), and their mixtures: Transactivation with human and rainbowfish hormone receptors and transcriptional effects in zebrafish (*Danio rerio*) eleuthero-emryos. *Aquat. Toxicol.* **2017**, *182* (November 2016), 142–162. https://doi.org/10.1016/j.aquatox.2016.11.001.

(59) Berggren, E.; Amcoff, P.; Benigni, R.; Blackburn, K.; Carney, E.; Cronin, M.; Deluyker, H.; Gautier, F.; Judson, R. S.; Kass, G. E. N.; Keller, D.; Knight, D.; Lilienblum, W.; Mahony, C.; Rusyn, I.; Schultz, T.; Schwarz, M.; Schüürmann, G.; White, A.; Burton, J.; Lostia, A.M.; Munn, S.; Worth, A. Chemical safety assessment using read-across: Assessing the use of novel testing methods to strengthen the evidence base for decision making. *Environ. Health Perspect.* **2015**, *123* (12), 1232–1240. https://doi.org/10.1289/ehp.1409342.